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Chapter 20

Transposon Insertion Mutagenesis for Archaeal Gene Discovery

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Abstract

Archaea constitute the third domain of life, but studies on their physiology and other features have lagged behind bacteria and eukarya, largely due to the challenging biology of archaea and concomitant difficulties in methods development. The use of genome-wide en masse insertion mutagenesis is one of the most efficient means to discover the genes behind various biological functions, and such a methodology is described in this chapter for a model archaeon *Haloferax volcanii*. The strategy successfully employs efficient in vitro transposition in combination with gene targeting in vivo via homologous recombination. The methodology is general and should be transferable to other archaeal species.

Key words Insertion mutant library, Gene discovery, *Haloferax volcanii*, Halophilic archaea, MuA transposase

1 Introduction

Archaea constitute the third domain of life, are ubiquitous in different types of environments, and often live in habitats with extremely harsh conditions. Thus, these organisms are biologically intriguing and potentially constitute an enormous resource for biotechnology applications. Yet, largely due to their challenging biology and concomitant difficulties in methods development, the biochemical pathways and genetic basis behind many unique archaeal features remain poorly characterized or entirely uncharacterized. Advanced methodologies are thus warranted for these organisms.

Insertion mutant libraries, which contain randomly distributed genomic alterations in each gene, provide a valuable resource for studies aimed at delineating molecular mechanisms behind biological functions. Such libraries have proven their immense usefulness for gene discovery, particularly in microbiological research. Until recently, archaea were underrepresented in such studies, as adequate insertion libraries had only been made for two

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archaeal species, the methanogens *Methanosarcina acetivorans* and *Methanococcus maripaludis* [1, 2].

Now, a recent study has widened the scope and applicability of insertion mutant libraries to halophilic archaea. The paper by Kiljunen et al. [3] describes a transposition-based method to generate a comprehensive insertion mutant library for the easily cultivable model archaeon, *Haloferax volcanii*, and moreover, the use of the library for gene discovery. The methodology used exploited a MuA-transposase-catalyzed in vitro transposition reaction and combined it with in vivo gene targeting by homologous recombination. As a result, a robust and widely applicable strategy was devised. The strategy entails the following steps: (1) Isolation of genomic DNA, its fragmentation, and tagging with a transposon; (2) Cloning of the tagged genomic fragments in *E. coli* to generate a plasmid library that covers the entire chromosome with overlapping fragments; (3) Amplification of the library, isolation of tagged chromosomal fragments, and their size selection; and (4) Transformation of the fragments into *H. volcanii* and en masse gene targeting via homologous recombination. The end product is a *H. volcanii* insertion-mutant library, in which each clone harbors a single transposon insertion in its genome. The library is an ideal resource for efficient gene discovery, and it facilitates the identification of nonessential genes behind any specific biochemical pathway. The strategy used for the construction of the library should readily be transferable to other archaeal species.

2 Materials

H. volcanii is sensitive to trace amounts of contaminants such as detergents as well as to impurities found in culture media. Therefore, it is advisable to rinse all glassware thoroughly with water and use for medium preparation *only* those commercial products specified in this protocol (*see* **Note 1**).

2.1 Equipment

1. Tabletop centrifuge that accommodates 50 ml centrifuge tubes.
2. 37 °C incubator and shaker.
3. Tabletop centrifuge (for 15 ml tubes).
4. Microcentrifuge.
5. Ultracentrifuge.
6. Heating block.
7. Water bath.
8. Equipment for ion-exchange chromatography.
9. Equipment for preparative agarose gel electrophoresis.
10. Electroporation apparatus and cuvettes.

11. 4.6 × 100 mm steel anion-exchange column, Gen-Pak FAX (Waters).

2.2 Media

1. 18% Modified Growth Medium (MGM): First, make a 30% salt water (SW) solution by dissolving 240 g NaCl, 30 g $MgCl_2 \cdot 6H_2O$, 35 g $MgSO_4 \cdot 7H_2O$, and 7 g KCl into ~800 ml water. Add slowly 5 ml 1 M $CaCl_2$. Adjust the pH to 7.5 with 1 M Tris–HCl, pH 7.5 and add water to a final volume of 1000 ml. For liquid 18% MGM, dissolve 5 g peptone (Oxoid) and 1 g yeast extract (Difco) into 600 ml SW and 367 ml water. Adjust the pH to 7.5 with 1 M Tris base and fill the volume to 1000 ml with water. For solid medium, add Bacto agar (Difco) 15 g/l [3] (*see* **Note 1**).
2. SOB medium: 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl. Autoclave.
3. SOC medium: Add to 100 ml of SOB solution 1 ml of 2 M $MgCl_2$ and 2 M glucose from stock solutions sterilized by filtration through a 0.22 μm filter.
4. Luria–Bertani medium (LB): 1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl. For solid medium, add Bacto agar to 1.5%. Supplement LB with ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) when needed.

2.3 Chemicals, Buffers

1. ST buffer: 1 M NaCl, 20 mM Tris–HCl, pH 7.5.
2. Saturated phenol, pH 7.9.
3. Chloroform.
4. Absolute ethanol.
5. 70% ethanol.
6. TE buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA.
7. 3 M sodium acetate, pH 7.0.
8. CsCl.

2.4 Molecular Biology Reagents

1. Lysis solution: 100 mM EDTA, pH 8.0, 0.2% SDS.
2. RNase A stock solution (10 mg/ml).
3. *Aci*I, *Hpa*I, *Taq*I, *Bgl*II, *Cla*I restriction enzymes.
4. Transposon carrier plasmid pMPH20 [4].
5. pBlueScriptSK+ plasmid.
6. Plasmid DNA isolation kit.
7. Alkaline phosphatase.
8. T4 DNA ligase.
9. *E. coli* electrocompetent DH10B cells. Stored at −70 °C.
10. *E. coli* DH5α electrocompetent cells. Stored at −70 °C.
11. Ethidium bromide.

2.5 DNA Transformation of H. volcanii

1. Buffered spheroplasting solution: 1 M NaCl, 27 mM KCl, 50 mM Tris–HCl, pH 8.5, 15% (w/v) sucrose [3].
2. Buffered spheroplasting solution with 15% glycerol: 1 M NaCl, 27 mM KCl, 50 mM Tris–HCl, pH 8.5, 15% (w/v) sucrose, 15% (v/v) glycerol [3].
3. 0.5 M EDTA, pH 8.0.
4. Unbuffered spheroplasting solution: 1 M NaCl, 27 mM KCl, 15% (w/v) sucrose. Adjust pH to 7.5 with 1 M NaOH (~10 µl/100 ml) [3].
5. 60% PEG 600 solution: 1500 µl PEG 600 (Merck) + 1000 µl unbuffered spheroplasting solution [3].
6. Spheroplast dilution solution: Dissolve 15 g sucrose in 76 ml 30% SW, and add water up to 100 ml. After autoclaving, add 0.75 ml of 0.5 M $CaCl_2$ [3].
7. Regeneration solution: First, make a 10× YPC solution by dissolving 1.25 g Yeast Extract (Difco), 0.25 g Peptone (Oxoid), and 0.25 g casamino acids (Difco) in ~19 ml water. Adjust the pH to 7.5 with 1 M KOH. Adjust the volume to 25 ml with distilled water. For the regeneration solution, dissolve 37.5 g sucrose in 150 ml 30% SW and 25 ml 10× YPC. Adjust the volume to 250 ml with distilled water. After autoclaving, add 1.5 ml of 0.5 M $CaCl_2$ [3].
8. Transformant dilution solution: Dissolve 37.5 g sucrose in 150 ml 30% SW and adjust the volume to 250 ml with water. After autoclaving, add 1.5 ml of 0.5 M $CaCl_2$ [3].
9. 80% glycerol-6% SW: Mix 80 ml glycerol and 20 ml 30% SW. After autoclaving, add 200 µl of 0.5 M $CaCl_2$ [3].
10. Hv-Ca: First, make a 10× Ca stock solution by dissolving 1.7 g casamino acids (Difco) in ~25 ml water. Add 800 µl of 1 M KOH and adjust the volume to 33 ml with water. To prepare Hv-Ca agar, measure into a 500 ml bottle 5 g agar (Difco), 100 ml of water and 200 ml of 30% SW. Boil to dissolve and add 10× Ca (33 ml). Autoclave. Cool to ~60 °C and add slowly 2 ml of 0.5 M $CaCl_2$ and 300 µl of a mixture of thiamine (0.89 mg/ml) and biotin (0.11 mg/ml). For strain *H. volcanii* H295 add 340 µl of uracil (50 mg/ml in DMSO) [3].

2.6 Mu In Vitro Transposition

1. 2× Mix: 50 mM Tris–HCl, pH 8.0, 200 µg/ml BSA (bovine serum albumin), 30% (w/v) glycerol. Use high-quality molecular biology grade BSA. Store at −70 °C.
2. Triton X-100: Make a 1.25% solution from a 10% stock solution by diluting with H_2O directly prior to use.
   - 2.5 M NaCl, 0.25 M $MgCl_2$

3. MuA transposase protein (Thermo Fisher Scientific) 220 ng/µl in MuA dilution buffer: 0.3 M NaCl, 25 mM Hepes, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol.

3 Methods

3.1 Transposon-Tagged Plasmid Library

For *H. volcanii* chromosomal DNA isolation and fragmentation, all procedures must be carried out at room temperature (RT) unless otherwise indicated.

1. Culture *H. volcanii* cells on 18% MGM agar dishes at 45 °C for 3–5 days (*see* **Note 2**). Inoculate 1–4 colonies into 5 ml of 18% MGM liquid medium and culture with shaking at 45 °C for ~32 h, until the late exponential culture phase is reached.
2. Collect cells from 3 ml of the cell suspension by microcentrifuging at 3500 × *g* for 8 min and resuspend the cells in 200 µl of ST buffer. Transfer the suspension into a 1.5 ml microtube. To lyse the cells, add 200 µl of lysis solution and mix carefully by gently inverting the tube (to avoid extra DNA shearing, do not vortex or pipette to mix).
3. Add 400 µl of phenol. Mix gently with a tabletop rotator for 30 min. Transfer the tube into a heat block (or water bath) and incubate at 60 °C for 1 h. To separate phases, use a microcentrifuge at maximum speed for 5 min, and transfer the viscous supernatant into another 1.5 ml microtube. Repeat the phenol extraction procedure but without the 60 °C incubation step. Extract the supernatant twice with 600 µl of chloroform.
4. Add 1 ml of ethanol and mix gently for a few minutes to precipitate DNA. Transfer the thread-like DNA precipitate (e.g., with a pipette tip) into a tube containing 1 ml of 70% ethanol. Microentrifuge at maximum speed for 10 min, remove the supernatant, and air-dry the DNA pellet. Dissolve DNA in 300–500 µl of TE buffer. Complete dissolution of the DNA may require up to 3 days (*see* **Note 3**).
5. Remove RNA by incubating with RNase A (at 0.2 mg/ml) for 30 min at 37 °C. Remove the enzyme by extracting with saturated phenol and twice with chloroform. Ethanol-precipitate the DNA with 1/10 volume of 3 M sodium acetate (pH 7.0). Dissolve the DNA in TE buffer as above.
6. Digest genomic DNA partially with three different enzymes (*Aci*I, *Hpa*II, and *Taq*I) in three separate reactions (*see* **Note 4**). Aim for a broad DNA fragment size distribution, in which a large proportion of the fragments falls within the size range of 2–4 kb. Remove the enzyme by phenol and chloroform extractions as in **step 5** above. Ethanol-precipitate and dissolve the DNA in water (*see* **Note 5**).

3.2 Transposon DNA (See Note 6)

1. TrpA-cat-Mu transposon DNA (*see* **Note 7**) is released from its carrier plasmid pMPH20 [4] by *Bgl*II digestion. The 2212 bp linear transposon can be purified in large quantities by the use of anion-exchange chromatography [6]. Preparative agarose gel electrophoresis is a convenient means to purify several micrograms of transposon DNA for a few experiments. Store the purified transposon DNA in TE buffer.

3.3 Mu In Vitro Transposition and Size-selection of the Reaction Products

1. Use DNA digested with each enzyme (*Aci*I, *Hpa*II, and *Taq*I) as a target in three separate transposition reactions. With each of them, assemble several standard in vitro DNA transposition reactions on ice as specified in Table 1 (*see* **Note 8**). Add MuA transposase as the last component just prior to incubation.
2. Incubate the reactions at 30 °C for 60 min and pool all the reaction products into one tube.
3. Extract once with saturated phenol and twice with chloroform. Ethanol-precipitate the DNA and dissolve in TE buffer.
4. Purify the 4–6 kb fragments using anion-exchange chromatography with a Gen-Pak FAX column (*see* **item 10** in Subheading 2.1 for the column). Use conditions described in the manufacturer's instructions.
5. As an alternative method, use preparative agarose gel electrophoresis for size selection and gel purification kits for DNA isolation.

3.4 Vector DNA

1. Isolate pBlueScript SK+ plasmid DNA (*see* **Note 9**) using any standard commercial plasmid isolation kit.
2. Digest 10 µg of pBlueScript SK+ plasmid DNA with *Cla*I restriction enzyme (4 U/µg) at 37 °C overnight.

Table 1
Mu in vitro transposition reaction mixture

Reagent	Standard reaction
2× mix	25 µl
Digested genomic DNA as target (800–1000 ng)	Typically 2–6 µl
TrpA-cat-Mu transposon as donor DNA (0.5 pmol/µl)	2 µl (1 pmol)
2.5 M NaCl	2 µl
1.25% Triton X-100 (freshly diluted)	2 µl
0.25 M $MgCl_2$	2 µl
H_2O	Up to 48 µl
MuA (220 ng/µl)	2 µl (440 ng, 5.4 pmol)
	Total 50 µl

3. Dephosphorylate the digested vector DNA with calf intestinal alkaline phosphatase as recommended by the supplier.
4. Isolate the dephosphorylated linear vector DNA using preparative agarose gel electrophoresis.

3.5 Plasmid Library Generation

1. Ligate the *Cla*I-digested vector with size-selected in vitro transposition reaction products using T4 DNA ligase and reaction conditions recommended by the supplier. Typically 50 ng of digested plasmid DNA is ligated with a 3-fold molar excess of DNA inserts in a reaction volume of 15 μl.
2. Thaw competent *E. coli* DH10B cells on ice (*see* **Note 10**).
3. Add 1 μl of ligation mixture into 25 μl of electrocompetent cells in a cold microtube. Transfer the mixture into an ice-cold electroporation cuvette (0.1 cm electrode spacing) (*see* **Note 11**).
4. Electroporate using the following pulse settings: voltage 1.8 kV, resistance 200 Ω, and capacitance 25 μF (*see* **Note 12**).
5. Add 1 ml SOC (room temperature solution), transfer into a microcentrifuge tube and incubate at 37 °C by shaking (220 rpm) for 30 min.
6. Spread the cells on LB culture plates containing ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) and incubate at 37 °C overnight.
7. Collect a suitable number of colonies (*see* **Note 13**) by adding per plate 1 ml LB medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) (LB-amp-cam), and scraping the colonies into a single pool (*see* **Note 14**). Add a suitable volume of fresh LB-amp-cam medium and grow at 37 °C by shaking (220 rpm) for 2.5 h (*see* **Note 15**).
8. Isolate plasmid DNA using a plasmid isolation kit of suitable capacity. This plasmid pool represents the primary plasmid library with the diversity defined by the number of the collected colonies used for its preparation (*see* **Note 16**).

3.6 Library Amplification and Purification (See Note 17)

1. Electrotransform aliquots from the primary plasmid library into *E. coli* DH5α, selecting for ampicillin and chloramphenicol resistance as above (*see* **Note 18**).
2. Collect a suitable number of colonies (*see* **Note 19**) and isolate plasmid DNA as above.
3. Purify supercoiled plasmid forms from the plasmid library by CsCl gradient ultracentrifugation (*see* **Note 20**). The published protocol is recommended [7].

3.7 H. volcanii Insertion Mutant Library

1. Use two different pairs of restriction endonucleases to release transposon-tagged *H. volcanii* DNA fragments from the plasmid library (*see* **Note 21**).

2. Isolate suitable-sized (4–6 kb) fragments using preparative agarose gel electrophoresis. Prepare enough purified DNA (several micrograms) to be used in the subsequent transformation step.

Perform the following steps at room temperature unless otherwise indicated.

3. To prepare *H. volcanii* competent cells (*see* **Notes 22** and **23**), inoculate 5 ml of 18% MGM medium with ~4 *H. volcanii* colonies and culture by shaking at 200 rpm, 45 °C for 24 h. Transfer 0.5 ml of the cell culture into 50 ml of fresh 18% MGM and culture as above for ~20 h until the absorbance (OD) at 600 nm is 0.8–1.0.
4. Divide the cell culture into two 25 ml aliquots in 50 ml centrifuge tubes and centrifuge in a table-top centrifuge (4500×*g*) for 10 min. Remove the supernatant and resuspend each pellet in 10 ml of buffered spheroplasting solution. Centrifuge as above and resuspend each pellet in 2.5 ml of buffered spheroplasting solution with 15% glycerol. Pool the suspensions and divide into suitable (e.g., 600 µl) aliquots. Flash-freeze the cells in liquid nitrogen and store at −80 °C. *H. volcanii* competent cells can be stored for several months. However, a decrease in the transformation efficiency is expected during prolonged storage (*see* **Note 24**).
5. Thaw 200 µl of competent *H. volcanii* cells for each transformation at room temperature and transfer them into a 2 ml centrifuge tube. Add 20 µl of 0.5 M EDTA, pH 8.0. Mix gently by inverting the tube and incubate for exactly 10 min to form spheroplasts.
6. Mix 10 µl (containing 1 µg) of DNA to be transformed, 15 µl unbuffered spheroplasting solution, and 5 µl 0.5 M EDTA, pH 8.0 (*see* **Note 25**).
7. Following the spheroplasting incubation (*see* **step 3** in Subheading 3.7), add the DNA mixture onto the tube wall and by gently rotating mix the solution with spheroplasts. Incubate for 5 min. Add 250 µl of freshly prepared 60% PEG 600 solution by slowly pipetting along the tube wall. Mix gently as above and incubate for 30 min.
8. Add 1.5 ml of spheroplast dilution solution and incubate for 2 min. Microcentrifuge at 3500×*g* for 10 min. Remove the supernatant. To regenerate, add 1 ml of regeneration solution gently onto the spheroplasts and incubate undisturbed at 45 °C for 1.5–2 h. Resuspend the cells and incubate with slow rotation (~100 rpm) at 45 °C for 4 h.
9. Microcentrifuge at 3500×*g* for 8 min. Remove the supernatant and resuspend into 1 ml of transformant dilution solution.

10. Plate the transformed cells directly onto appropriate selection plates or freeze the suspension for later use (*see* **Note 26**).
11. To store the cell suspension, add 335 µl of 80% glycerol-6% SW, divide into suitable (e.g., 200 µl) aliquots, flash-freeze in liquid nitrogen, and transfer for storage at −80 °C.
12. Spread the cells onto appropriate selective plates as follows (*see* **Note 27**). Thaw the frozen cells at room temperature and spread (with a suitable dilution) onto a selection plate. Use for example, 100 µl per standard petri dish and culture at 45 °C for 1 week. Note that some insertion mutants have a reduced growth rate, resulting in variable colony sizes among the member clones of the library.

3.8 Library Validation

1. Validate the library with regard to the insertion copy number (*see* **Note 28**).
2. Determine the insertion site of the transposon in the genome of a library clone by DNA sequencing (*see* **Note 29**).

4 Notes

1. It has been observed that the quality of media components and reagents may differ substantially among manufacturers, and certain impurities inhibit the growth of haloarchaeal cells. More details about the purity requirements can be found in the Halohandbook [3].
2. Other rich media can be used. More alternatives can be found in Halohandbook [3].
3. Alternatively, a genomic DNA isolation kit may be used. Most commercial kits should be suitable for *H. volcanii* DNA isolation.
4. We recommend partial digestions for DNA fragmentation and the usage of several enzymes. This guarantees an extensive coverage of the genome with overlapping fragments and in practice eliminates any potential bias caused by restriction site distribution. The enzymes recommended in this protocol (*Aci*I, *Hpa*II, *Taq*I) each recognize a 4-bp sequence and generate a protruding 5'-GC overhang that is compatible with a *Cla*I site in cloning. The conditions for partial digestions need to be adjusted for each restriction enzyme separately. It is advisable to use otherwise constant reaction conditions but different amounts of enzyme. The size distribution of DNA fragments can be analyzed by the use of standard agarose gel electrophoresis.
5. It is advisable to use 5–10 µg of genomic DNA for digestions. It is important to dissolve the digested DNA fragments in

water, as extra salt is inhibitory in the subsequent Mu in vitro DNA transposition reaction. The concentration of the fragmented DNA should preferably be ≥200 ng/μl.

6. Mini-Mu transposons utilized in in vitro reactions are linear DNA molecules that contain at each of their ends, in an inverted relative orientation, a 50-bp segment from the right end of the phage Mu genome. This so-called R-end segment contains a pair of MuA transposase-binding sites. The DNA between the R-ends can be of any origin and modified with regard to the needs of a particular application.
7. The TrpA-cat-Mu transposon [4] contains two selectable markers: *cat* for *E. coli* and *trpA* for *H. volcanii*. It is maintained within its carrier plasmid pMPH20 that can be obtained by request from *H. savilahti*.
8. Material from three to six reactions per enzyme digestion should yield enough DNA for the subsequent cloning step.
9. Other standard *E. coli* cloning vectors may be used. However, a suitable vector needs to harbor a unique *Cla*I site, which is flanked by at least two pairs of unique restriction sites not present in the TrpA-cat-Mu transposon (the transposon sequence is available upon request).
10. Although several *E. coli* strains may be used for cloning, DH10B is recommended as it yields good quality plasmid DNA and can be electroporated efficiently. High efficiency electrocompetent cells can be prepared using the protocol described [5], or they may be obtained from commercial vendors.
11. Perform several parallel electroporations to yield a desired number of transformants for the generation of a plasmid library. The number defines the maximum diversity of potential transposon insertion sites within this library.
12. The protocol has been developed for Gene Pulser II electroporation apparatus (Bio-Rad). If another brand is used the optimal pulse parameters may differ.
13. The desired colony number (*see* **Note 11**) varies according to the gene number of the target organism. A tenfold excess over the gene number is recommended, as it guarantees a comprehensive library, in which each gene will be tagged with a very high likelihood. For the calculation of the probability *see* Kiljunen et al. [4].
14. Standard (diameter 9 cm) plates can accommodate up to 1000 separate colonies, although this number may vary among different strains. If larger plates are used, the volume of the medium added should be increased proportionally. For example, 10 ml is suitable for large (25 × 25 cm) square plates.

15. The volume recommended depends on the number of collected colonies. For example, 200 ml of medium may be used for 40,000 colonies.
16. It is advisable to minimize the time used for liquid cultures, as the growth rate of the clones may differ. Accordingly, longer culture times may bias the original plasmid diversity.
17. Amplification of the plasmid library guarantees a sufficient amount of plasmid DNA for the purification by CsCl gradient ultracentrifugation.
18. Standard chemical transformation may also be used here given the efficiency is high enough to obtain the desired number of transformants. Although several *E. coli* strains may be used, DH5α is recommended. This strain yields very high quality plasmid DNA with the majority of the molecules being supercoiled.
19. To retain the original plasmid diversity with a very high probability, collect at least 20 times more colonies than what was collected for the primary plasmid library. Notice that you will need a substantial amount of DNA for the next step. We recommend isolating 1–2 mg plasmid DNA at this stage.
20. Alkaline plasmid preparation methods produce a fraction of collapsed supercoiled plasmid forms, which enter the cells efficiently and cannot be digested with restriction endonucleases. It is important to remove them, as they would generate false positive colonies upon transformation into *H. volcanii* (*see* ref. 4 for more details). CsCl gradient ultracentrifugation is a recommended means to remove collapsed supercoiled plasmid forms.
21. In the study of Kiljunen et al. [4], pBlueScript SK+ plasmid was used as the cloning vector. The restriction enzyme pairs used for the successful fragment release in that study were *Xho*I/*Hin*dIII and *Kpn*I/*Eco*RV. It is advisable to digest a substantial amount of DNA at this stage (e.g., 100–200 μg). Use conditions recommended by the enzyme supplier.
22. In principle, any *H. volcanii* strain could be used. We used the strain H295, which is devoid of Mre11 and Rad50. Due to these deficiencies, its homologous recombination activity is increased 100-fold [8].
23. More information about *Haloferax* transformation procedures can be found in Halohandbook [3].
24. Transformation efficiency of competent cells can be tested with any *H. volcanii* plasmid and appropriate selection plate.
25. DNA may be in any commonly used buffer (such as TE) or in water.
26. The expected transformation efficiency is 10^4–10^5 cfu per microgram of transformed DNA.

27. With the tryptophan auxotrophy marker gene (*TrpA*), the selection plate used is Hv-Ca. See Halohandbook for more information about selection plates and the required additives [3].
28. A genome-wide insertion mutant library used as a gene discovery tool should contain mutants with single-copy insertions. Southern hybridization or quantitative PCR can be used to evaluate this. In the protocol described, the stoichiometry in the transformation step favors single-copy genomic integrations, i.e., 1 out of 2000 target cells becomes transformed [4]. Thus, the vast majority of the clones in the library is expected to contain a single genomic insertion.
29. The transposon insertion site in the genome of a mutant can be determined by initially cloning the transposon with its genomic flanks from the chromosomal DNA using a restriction enzyme that does not cut within the transposon DNA. The sequences bordering the transposon DNA are then determined using standard Sanger sequencing [5]. However, with the fast development of next-generation sequencing (NGS) techniques, whole-genome sequencing by NGS may soon be the fastest and most cost-effective way to determine the insertion site in a microbial genome.

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